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Balanites aegyptiaca ameliorates insulin secretion and decreases pancreatic apoptosis in diabetic rats: Role of SAPK/JNK pathway

Running title: *Balanites aegyptiaca* inhibits SAPK/JNK in diabetic rats

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Key words: *Balanites aegyptiaca*, Diabetes, SAPK-JNK pathway, Oxidative stress, Apoptosis

Abstract

SAPK-JNK pathway performs a significant role in the pathogenesis of type 2 diabetes. *Balanites aegyptiaca* (BA) is used as an anti-diabetic agent in folk medicine however its hypoglycemic mechanism is not fully elucidated. The current study aimed to evaluate the effect of crude extract, butanol, and dichloromethane fractions from BA on the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK-JNK) pathway in experimental diabetic rats. Six groups of male Wistar rats were included: normal control, diabetic, diabetic rats treated with crude, butanol or dichloromethane fraction from BA (50 mg/kg BW) and diabetic rats treated with gliclazide as a reference drug for one month. Our results suggested a protective role of treatment of diabetic rats with BA against oxidative stress-induced SAPK-JNK pathway. Moreover, BA treatment produced a reduction in plasma glucose, HbA_{1c}, lactic acid, lipid profile, malondialdehyde levels and produced an increase in insulin, reduced glutathione levels, catalase and superoxide dismutase activities compared with untreated diabetic rats. Moreover, it decreased apoptosis signal-regulating kinase 1, c-Jun N-terminal kinase 1, protein 53 and increased insulin receptor substrate 1 in rat pancreas while it increased glucose transporter 4 in rat muscle. Analysis of BA extracts by LC-HRMS revealed the presence of different saponins with reported hypoglycemic effect. In conclusion, BA exerted hypoglycemic, hypolipidemic, insulinotropic and antioxidant effects. Additionally, it reduced apoptosis in pancreatic β-cells and increased glucose uptake in muscle. These results suggest that the hypoglycemic effect of BA is due to the inhibition of the SAPK-JNK pathway.

Key words: *Balanites aegyptiaca*, Diabetes, SAPK-JNK pathway, Oxidative stress, Apoptosis

List of Abbreviations

ASK1	apoptosis signal-regulating kinase 1
BA	Balanites aegyptiaca
BBF	Balanites butanol fraction
BCE	Balanites crude extract
BDF	Balanites dichloromethane fraction
DM	diabetes mellitus
FPG	fasting plasma glucose
GSH	reduced glutathione
JNK1	c-Jun N-terminal kinase 1
LA	Lactic acid
MDA	malondialdehyde
p38 MAPK	p38 mitogen-activated protein kinase
p53	the tumor suppressor protein 53
PAS	periodic acid-Schiff
SAPK-JNK	stress-activated protein kinase- c-Jun N-terminal kinase
SOD	superoxide dismutase
STZ	streptozotocin
T2DM	type 2 diabetes mellitus
TC	total cholesterol
TG	triglycerides

1. Introduction

Diabetes mellitus' (DM) worldwide occurrence is skyrocketing as a result of population aging and unhealthy lifestyle [1]. Type 2 diabetes mellitus (T2DM) has been reported as the most common disease form, affecting about 90% of diabetic patients. DM is one of the leading morbidity reason, causing microvascular and macrovascular complications [2]. The main diabetes risk factor is oxidative stress. It changes insulin sensitivity either by increasing insulin resistance or impairing glucose tolerance. The mechanisms for both are multifactorial and complex, including numerous cell signaling pathways like the c-Jun N-terminal kinase (JNK) [known as the stress-activated protein kinase (SAPK)], p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C (PKC) pathways, which are triggered by oxidative stress in several cells, including pancreatic β-cells [3]. The SAPK-JNK triggering inhibits insulin synthesis and affects insulin action. This pathway inhibition in diabetic mice improves insulin resistance and glucose tolerance. The SAPK-JNK pathway plays an essential role in T2DM pathogenesis and would be a target for diabetes treatment [4]. DM treatment protocols consist of diet, insulin and oral hypoglycemic drugs. Although treatments can control numerous diabetes features, complications are common and the mortality index continues to rise [5], so new drugs are indeed required.

One of the first recorded treatments for DM, derived from medicinal plants, is described in the Ebers papyrus (c. 1550 BC) [6]. *Balanites aegyptiaca* Del (family Zygophyllaceae) was identified as the desert date in English and heglig or balah el-abeed in Arabic. *Balanites aegyptiaca* (BA) is native to Africa and the Middle East. The BA fruits are used as an oral hypoglycemic agent for diabetes treatment in Egyptian folk medicine [7]. In this work, we have evaluated the chemical profile of three fractional extracts [crude, butanol (polar fraction), dichloromethane (non-polar fraction)] of BA fruits for their antidiabetic potential. To the best of our knowledge, research about the BA hypoglycemic mechanism is scant, therefore more research is needed in this area. We hypothesized that the BA hypoglycemic effect is partly due to the inhibition of the SAPK-JNK pathway and the antioxidant effect on the other part. This study aimed to address the role of BA on experimentally streptozotocin-induced diabetes in albino Wistar rats through studying the SAPK-JNK pathway.

2. Materials and Methods

2.1 Chemicals

Extraction solvents (ethanol, butanol, and dichloromethane) were purchased from El Nasr pharmaceutical chemical Co. (Cairo, Egypt). Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and glucose, lipid profile and oxidative stress parameters kits were purchased from Biodiagnostic (Giza, Egypt). Glycated hemoglobin (HbA_{1C}) kit was purchased from Sigma Diagnostics (Budapest, Hungary). Insulin kit was purchased from DRG® International (Mountainside, N.Y., USA). Lactic acid (LA) and apoptosis signal-regulating kinase 1 (ASK1) kits were purchased from MyBiosource (San Diego, USA). c-Jun N-terminal kinase 1 (JNK1) kit was purchased from Wuhan Fine Biological Technology (Hubei, China). The tumor suppressor protein 53 (p53) kit was purchased from RayBiotech (Atlanta, USA). Insulin receptor substrate 1 (IRS1) and glucose transporter 4 (GLUT4) kits were purchased from Elabscience Biotechnology (Hubei, China).

2.2 Plant materials

The BA fruits were purchased from Haraz for herbs, oils and natural extracts (Cairo, Egypt). The BA fruits were authenticated by Prof. Dr. Ahmed Mohamed Fawzi in Ministry of Agriculture and Land Reclamation. The date is dark brown, and both unripe and ripe fruits fleshy pulps are edible and eaten dried or fresh. Fruit fleshes were sliced and weighed and the seeds were discarded.

2.2.1 Extracts preparation

The BA fruit fleshes (2 Kg) were extracted by cold maceration using 70% ethanol to give the residue (62 g). The BCE was fractionated by liquid-liquid extraction with dichloromethane and *n*-butanol and evaporated to give nonpolar and polar residues, respectively.

2.2.2 LC-HRMS analysis

High resolution mass spectrometric data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA auto sampler, and Accela pump). The following conditions were

applied: capillary voltage 45 V, capillary temperature 260°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30 000). For LC/MS, a Sunfire C18 analytical HPLC column (5 μm, 4.6 mm × 150 mm) was used with a mobile phase of 0 to 100% MeOH over 30 minutes at a flow rate of 1 ml min^{-1}. The exact mass obtained for eluted peaks was used to deduce the possible molecular formulae for such mass, and these formulae were searched in Dictionary of Natural Products, CRC press, online version, for matching chemical structures. Analysis of data was performed using Xcalibur 3.0 and dereplication using the dictionary of natural products database V. 23.1 on DVD, Table (1) and Figure (1).

2.3 Animals and Experimental design

Sixty male Wistar albino rats weighing 100-120 g were purchased from National Research Centre (Cairo, Egypt). The rats were kept in a controlled environment and were allowed free accesses to standard chow diet and tap water and housed in polyacrylic cages. Rats were fasted overnight and were given a single STZ dose dissolved in cold citrate buffer (0.1M, pH 4.5) at 50 mg/kg BW, intraperitoneally (i.p), for T2DM induction [8]. Rats with fasting plasma glucose (FPG) level above 200 mg/dl after 72 hours were considered as diabetic [9] along with normal control. Rats were randomly divided into six groups (10 rats each) as follows:

Group1 (control): normal healthy rats were orally given saline by rat oral gavage and injected i.p. with a single dose of citrate buffer (0.1M, pH 4.5).

Group 2 (STZ): rats received a single STZ dose i.p (50 mg/kg BW).

Group 3 (STZ + Balanites crude extract (BCE)): rats received STZ as mentioned in group 2 and treated orally with BCE 50 mg/kg/day for one month.

Group 4 (STZ + Balanites butanol fraction (BBF)): rats received STZ as mentioned in group 2 and treated orally with BBF 50 mg/kg/day for one month.

Group 5 (STZ + Balanites dichloromethane fraction (BDF)): rats received STZ as mentioned in group 2 and treated orally with BDF 50 mg/kg/day for one month.

Group 6 (STZ + gliclazide (reference drug)): rats received STZ as mentioned in group 2 and treated orally with gliclazide 10 mg/kg/day for one month.

At the end of the experimental period, animals from the normal and the five diabetic groups were food deprived for 12-14 hours in order to withdraw blood from retro-orbital plexus for biochemical analysis, and then rats were killed by cervical dislocation.

2.4 Sample collection

Blood was collected in fluoride tubes where they were centrifuged at 3000 rpm for 15 minutes to obtain plasma for FPG estimation. Another blood sample was collected in EDTA tubes and was divided into two aliquots. The first aliquot was used for HbA_{1c} estimation. The second aliquot was centrifuged at 3000 rpm for 15 minutes in order to obtain plasma which in turn was divided into 2 parts. The first part was used for complete lipid profile estimation while the other part was stored at -80°C until insulin and LA assay using ELISA kits. Liver, skeletal muscles and pancreas were dissected, washed with cold phosphate buffer saline (PBS), and blotted dry with filter paper. Liver (1 g) was homogenized in 5 ml PBS using tissue homogenizer (Yellow line DI 18 Basic, Deutschland, Germany). The liver homogenates were centrifuged at 4000 rpm for 15 minutes. The supernatants were collected and used directly for the estimation of malondialdehyde (MDA), reduced glutathione (GSH) levels, catalase and superoxide dismutase (SOD) activities. The pancreas was preserved at -80˚C for ASK1, JNK1, p53, and IRS1 estimation. The skeletal muscle was preserved at -80˚C until the GLUT4 determination. The study was approved by our institutional animal ethics committee of Beni-Suef University and all procedures for agent administration, blood, and tissue collection were in accordance with the 8^{th} edition of the Guide for the Care and Use of Laboratory Animals published in 2011 by the United States National Academy of Sciences.

2.5 Methods

2.5.1 FPG and HbA_{1c} determination

Fasting plasma glucose level was determined using colorimetric kit purchased from Biodiagnostic (Giza, Egypt). HbA_{1c} was determined using colorimetric kit purchased from Sigma diagnostics (Budapest, Hungary).

2.5.2 Insulin and LA determination

Plasma Insulin level was estimated using insulin rat ELISA kit obtained from DRG® International (Mountainside, N.Y., USA) according to the manufacturer's instructions. LA concentration was estimated using rat lactic acid ELISA kit obtained from MyBiosource (San Diego, USA) according to the manufacturer's instructions.

2.5.3 Lipid profile determination

Total cholesterol (TC), HDL-C, LDL-C, and triglycerides (TG) were determined using colorimetric kits purchased from Biodiagnostic (Giza, Egypt).

2.5.4 Oxidative stress parameters determination

Catalase and SOD activities, MDA and GSH kits were purchased from Biodiagnostic (Giza, Egypt) and were determined in liver tissue homogenate according to the manufacturer's instructions.

2.5.5 Pancreatic ASK1 determination

The ASK1 was determined using rat ASK1 ELISA Kit obtained from MyBiosource (San Diego, USA) according to the manufacturer's instructions.

2.5.6 Pancreatic JNK1 and IRS1 determination

The JNK1 and IRS1 were determined using rat JNK1 and rat IRS1 ELISA kits obtained from Wuhan Fine Biological Technology (Hubei, China) and Elabscience Biotechnology (Hubei, China), respectively according to the manufacturer's instructions.

2.5.7 Pancreatic p53 determination

The p53 was determined using RayBio® rat p53 ELISA kit obtained from RayBiotech (Atlanta, USA) according to the manufacturer's instructions.

2.5.8 Muscular GLUT4 determination

The GLUT4 was determined using rat GLUT4 ELISA kit obtained from Elabscience Biotechnology (Hubei, China) according to the manufacturer's instructions.

2.5.9 Histopathological examination

Three animals were killed from each group; the pancreas and liver were collected, washed with saline and immersed in 10% buffered formalin. Liver, kidney, and pancreas samples were processed by paraffin technique. The tissue samples were dehydrated by ascending alcohols grades, cleared in xylene, impregnated in soft paraffin, embedded and blocked in hard paraffin. The blocks were cut by microtome 4-5 µm thickness and mounted on clean and dry glass slides.

The obtained slides were stained by:

1- Haemotoxylin and Eosin (H&E) general stain.
2- Periodic acid-Schiff (PAS) for liver glycogen demonstration.
3- Aldehyde Fuchsin for β-cell granules demonstration.

All techniques and stains were used according to Bancroft and Gamble (2008) [10] methods.

Additionally, measurement of the diameter of Langerhans' islets and the β-cells counting of rats' pancreas were performed in three fields for each section (i.e. nine fields for each group). The injury in β-cells of pancreas (stained by H&E) was scored in terms of the degree of cell damage, according to Gibson-Corley *et al.* (2013) [11] by the aid of Image J analysis software program (NIH, Bethesda, Maryland), using LEICA (DFC290 HD system digital camera, Heerbrugg, Switzerland) connected to the light microscope using 10× objective lens.

2.5.10 Statistical Analysis:

The data were expressed as a mean ± SD. The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey multiple comparison post-hoc test. The *p*

values less than 0.05 were considered significant. All calculations were made using Statistical Package for the Social Sciences 22 (SPSS, Chicago, Illinois, USA).

3. Results

3.1 LC-HRMS analysis results

Figure (1) and Table (1) showed similarities in different composition pattern between the crude, DCM, and butanol fractions. A total of twenty-three components were tentatively identified in the three extracts with some components present in one extract and absent in the others.

Similar activities exhibited in BCE and BBF could be explained due to the presence of components found exclusively in these two extracts. Balanitin-2, hexadecenoic acid, methyl protodioscin and 26-(O-β-D-glucopyranosyl)-3-β-[4-O-(β-D-glucopyranosyl)-2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-22,26-dihydroxyfurost-5-ene were present only in BCE and BBF while balanitin-1 and trigonelloside C were present in the three extracts.

3.2 Biochemical parameters

3.2.1 FPG and HbA_{1C}

Streptozotocin significantly increased FPG and HbA_{1c} levels compared to the normal group. In all treated groups, BA extracts significantly lowered FPG compared with the STZ group. The lowest FPG level was present in BBF and BCE groups, placing them below the gliclazide group. Moreover, BA extracts significantly reduced HbA_{1c} level compared with STZ and gliclazide groups (Table 2).

3.2.2 Plasma insulin and LA

Streptozotocin significantly decreased insulin levels compared with the normal and BA extracts groups. BBF significantly increased insulin level compared with the STZ, BCE, BDF, and gliclazide groups. The order of increasing insulin levels was BBF > BCE > gliclazide > BDF > STZ. Additionally, STZ significantly elevated LA level compared with the normal group. Treatment with BA extracts significantly decreased LA level compared with the STZ group. Surprisingly, BBF normalized LA level which did not occur in other groups (Table 2).

3.2.3 Plasma lipid profile

Treatment with BA extracts or gliclazide significantly decreased TC level compared with the STZ group. Moreover, BA extracts and gliclazide were succesfull in decreasing the higher TG level, especially those of BCE and BBF, which were comparable to normal levels. BDF and gliclazide groups TG levels were higher than that of the normal group. On the other hand, BA extracts significantly increased HDL-C compared to the STZ group. Gliclazide showed the least increase in HDL-C compared with BA extracts. BA extracts significantly decreased LDL-C and this effect appeared in the BBF and BCE groups, while gliclazide failed to decrease the high LDL-C level.

3.2.4 Atherogenic index

The BA extracts significantly decreased atherogenic index (LDL-C/HDL-C) compared to the STZ and gliclazide groups. Moreover, BA extracts restored atherogenic index to the normal value which did not occur in case of gliclazide.

3.2.5 Oxidative stress and antioxidant parameters

3.2.5.1 Liver MDA

Streptozotocin significantly elevated MDA level compared to the normal control. Treatment with BA extracts or gliclazide significantly decreased MDA level compared to the STZ group and the lowest MDA levels were present in the three BA extract-treated groups (Table 2).

3.2.5.2 Liver catalase and SOD activities

Streptozotocin significantly decreased catalase and SOD activities compared to the normal control. The catalase activity in the BBF group was comparable to that of the normal group and higher than that in BDF. Moreover, catalase activity was significantly higher in the three BA extracts compared to the gliclazide group. Additionally, BBF significantly increased SOD activity compared to the normal control, the other two extracts or gliclazide groups. The least increment in SOD activity was found in the BDF group. Gliclazide normalized the SOD activity placing it significantly lower than that of the BBF or BCE treated groups and higher than that of the BDF treated group (Table 2).

3.2.5.3 Liver GSH

Streptozotocin significantly reduced GSH level compared to the normal group. BA extracts significantly increased GSH level compared to the STZ group. Although, BDF showed the least increase in GSH level compared to that was noticed by the other two extracts, the three BA extracts were superior in increasing GSH level compared to gliclazide (Table 2).

3.2.6 SAPK/JNK pathway parameters

3.2.6.1 Pancreas ASK1, JNK1, and p53

Streptozotocin significantly elevated ASK1, JNK1, and p53 levels compared to the normal group. BA extracts significantly reduced the STZ-induced elevated levels of ASK1, JNK1, and p53 (Table 2).

3.2.6.2 Pancreas IRS1

Streptozotocin significantly reduced IRS1 level compared to the normal group. BA extracts significantly elevated the decreased IRS1 level induced by STZ. Moreover, the IRS1 level was significantly higher in the BCE group compared to the gliclazide group (Table 2).

3.2.7 Skeletal muscles GLUT4

Streptozotocin significantly reduced GLUT4 level compared to the normal group. BCE, BBF, and gliclazide groups significantly elevated GLUT4 level compared to the STZ group and the highest increase was present in the BCE group. BDF failed to increase GLUT4 compared to the STZ group (Table 2).

4. Discussion

This study addressed the role of BA on experimentally STZ-induced diabetes in rats through studying the SAPK-JNK pathway. The trademark of DM is the incapability to control glucose levels. The LC-HRMS analysis of BCE, BBF and BDF revealed the presence of Balanitin-1 and -2, diosgenin, stigmast-4-en-3-ol and pregn-5-ene-3β,16β,20(R)-triol-3-*O*-β-D-glucopyranoside. The last two compounds in addition to 26-(*O*-β-D-glucopyranosyl)-22-*O*-methylfurost-5-ene-3β,26-diol-3-*O*-β-ᴅ-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside

have been recently isolated and identified by Ezzat *et al.* (2017) [12] using ESI-MS in the methanol extract of BA. Another recent study by Rashad *et al.* (2017) [13] identified 13 compounds including phenolics, steroidal saponins, and a sterol glucoside in ethanol extract of BA using ultra-performance electrospray ionization-mass spectroscopy (UPLC-ESI-MS/MS). These results showed similarities such as the steroidal saponins (e.g. diosgenin) that had been identified in our study, and differed in some others, especially the phenolic compounds. Balanitin-1 and -2 were reported to have anti-inflammatory properties [14] and antidiabetic properties, especially balanitin-2 [7]. Diosgenin showed a reported hypocholesterolemic effect [15]. It also reduced endoplasmic reticulum (ER) stress and oxidative stress-mediated damage in diabetic rats pancreas [16]. Diosgenin acted as a hypoglycemic compound compared to glibenclamide [17]. Stigmast-4-en-3-ol produced significant hypoglycemic activity as well [18]. In the current study, BA extracts significantly reduced FPG and HbA_{1C}. These results are in agreement with those reported in diabetic rats [12] as well as in type 2 diabetic patients [13]. These effects could be attributed to the high content of bioactive compounds (diosgenin, stigmast-4-en-3-ol, and balanitin-2). Pure saponins extracted from the BA fruit mesocarp have been reported as a hypoglycemic agent when tested in rats in different concentrations and compared to glibenclamide. Saponins also inhibited *Escherichia coli* growth in rats. The main saponin compositions are Balanitin 1-3 [7]. Our results showed that the BBF and to some extent the BCE were more active than the BDF. This could be attributed to the presence of balanitin-2 only in the BCE and BBF, compared to balanitin-1, where it was present in the three fractions. Our LC-HRMS results highlighted the absence of trigonelline in all extracts. This is not in agreement to what has been previously reported by Farid *et al.* (2002) [19] and Farag *et al.* (2015) [20]. Thus, our findings suggested that the antidiabetic activity is mainly due to the saponin constituents, especially balanitin-2. The BBF was more antidiabetic than the BCE which could be attributed to the higher percentage of saponins, especially balanitin-2 in butanol fractions.

Streptozotocin treated rat model showed the partial or total destruction of pancreas β-cells with insulin drop, due to its reduced production [21]. BBF treatment increased plasma insulin level more than gliclazide suggesting the activation of some pancreas β-cells as previously reported by Ezzat *et al.* (2017) [12], who attributed the effect of insulin increment to furostanol saponin, namely 26-(*O*-β-D-glucopyranosyl)-22-*O*-methylfurost-5-ene-3β,26-diol-3-*O*-β-D-

glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside. On the contrary, Rashad *et al.* (2017) reported no effect on plasma insulin and C-peptide levels in type 2 diabetic patients treated with BA capsules although they identified the previous furostanol saponin along with another 12 compounds [13].

Diabetic rats showed increased LA due to augmented lipolysis and glycolysis, which is similar to ketoacidosis and lactic acidosis in diabetic patients [22]. BBF reduced LA level and therefore decreased the possibility of lactic acidosis.

In diabetes, the high plasma glucose level is accompanied by an escalation of TC, TG, LDL-C, atherogenic index and reduction in HDL [23]. In the present study, BA extracts lowered TC, TG, LDL-C, atherogenic index and increased HDL-C. These results are in agreement with that reported by Rashad *et al.* (2017) [13] who tested BA in type 2 diabetic patients. On the other hand, Ezzat *et al.* (2017) [12] reported a reduction of cholesterol level but no significant change in triglycerides level in BA-treated diabetic rats. The main risk factors for premature atherosclerosis are diabetes and hyperlipidemia and the cholesterol present in atherosclerosis plaque is a resultant of circulatory cholesterol [24], BA hypolipidemic and hypocholesterolemic effects could have a potential healing effect.

Hyperglycemia elevates free radical and increases lipid peroxidation (thiobarbituric acid reactive substances and hydroperoxides). According to Randle's glucose-fatty acid hypothesis, excessive free fatty acid released from adipose tissue for oxidation causes metabolites production that inhibits glucose utilization by tissues. These metabolites, which are implicated in the glucose-fatty acid cycle, are reactive oxygen species and hydrogen peroxide [25]. These substances cause damage to cellular structures and impair glucose metabolism. Elevated free radical concentration and lipid peroxidation decrease the antioxidant defense. This study supports the hypothesis that lipid peroxidation plays a role in diabetic complications.

Important antioxidants are catalase, GSH, and SOD. Catalase is enzyme dispersed in all animal tissues with its highest activity found in red blood cells and the liver. Catalase disintegrates hydrogen peroxide and protects tissues from highly reactive hydroxyl radicals [26]. Catalase activity is reduced during diabetes and this may result in deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides. BBF significantly elevated catalase

activity which revealed the antioxidant effect of BBF prohibiting glycation and enzyme inactivation *via* decreasing FPG. Inhibition of possible glycation of antioxidant enzymes and the resulting increase in activity had been reported with other plants, such as *Gongronema latifolium* and *Eugenia jambolana* recognized for their antidiabetic activity [27,28]**.**

Superoxide dismutase guards tissues against oxygen free radicals and transforms superoxides to hydrogen peroxide and inhibits damage to the biological system [29]. In our study, BA extracts decreased diabetes-induced oxidative stress and this decreased oxidative stress associated with elevated SOD activity.

Diabetic rats have decreased GSH levels. GSH has a multifaceted role in antioxidant defense. It could directly scavenge free radicals or indirectly detoxify reactive species through glutathione S-transferase and glutathione peroxidase activities [30]. BA extracts improved antioxidant status as demonstrated by elevated GSH level thereby adding to its protection from diabetic damage. Extracts could either increase GSH biosynthesis or reduce the oxidative stress leading to less GSH degradation or have both effects.

The liver's MDA levels of diabetic rats were significantly increased. BA extracts significantly reduced MDA, attributing to increased antioxidants levels which fight free radicals [31].

The SAPK-JNK is one of the most investigated pathways in obesity models of insulin resistance. JNK1 causes insulin resistance by direct inhibitory phosphorylation of IRS 1 and 2 [32]. T2DM is distinguished by pancreatic β-cell dysfunction and low-grade inflammation. Latest studies proposed that ASK1 is implicated in β-cell death [33]. The strongest ASK1 activator is oxidative stress, which is essential for oxidative stress-induced apoptosis through the MKK4/MKK7-JNK cascade activation [34]. In diabetic rats, ASK1 was significantly increased. BA extracts significantly reduced ASK1. These results suggested that BA extracts could reduce β-cell death, an observation that was supported by the histopathological study of the pancreas (Figure 2 and Table 3). The convergence of chronic ER stress towards pancreatic apoptosis was indicated in our study by increased JNK1 in diabetic rats' pancreas, which was significantly reduced by administration of BA extracts.

The transcription factor, p53 also induced pancreatic apoptosis [35]. P53 was elevated in diabetic rats but treatment with BA extracts decreased p53, which provided more evidence that BA extracts could help in reduction β-cell death.

The histopathological investigations demonstrated that BA extracts, especially BBF significantly increased the size of pancreatic islets and the number of cells when compared to the STZ group (Figure 2 and Table 3). These results could be attributed to lowering ASK1, JNK1 and p53 levels which led to reducing β-cell apoptosis in pancreatic islets, increased the insulin-positive β-cells number in the islets, promoted islet β-cell survival, and preserved islet mass. The histopathology of BA extracts treated rats' liver and kidney (data not shown) revealed no histopathological alterations of these two organs indicating that BA extracts did not disturb their normal functions.

Insulin produces its effects by binding to its specific receptor and the activated insulin receptor complex phosphorylates IRS1, which then binds other signaling molecules to propagate the insulin signal [36]. Administration of STZ to rats significantly decreased pancreas IRS1 which agreed with a previous study that showed decreased IRS1 in DM [37]. Treatment with BA extracts minimized the reduction in pancreas IRS1 of these rats. These results could suggest that BA extracts have hypoglycemic effects through an insulinotropic pathway in diabetic rats. T2DM is characterized by insulin resistance and insulin deficiency, leading to hyperglycemia with high morbidity and mortality rates [38].

Despite extensive investigations into the mechanism of T2DM, insulin resistance is still the core of our understanding of the etiology of this disease. Rats administered with STZ had significantly increased FPG and decreased GLUT4, which were similar to those in human T2DM. Now, 14 types of glucose transport protein have been revealed, each having purposes with various distributions. GLUT4 is important for sustaining glucose metabolism homeostasis and insulin sensitivity since it is associated with glucose transport into myocytes and adipocytes in response to insulin or exercise stimuli [39]. Skeletal muscle is credited for insulin-stimulated glucose uptake and lipid oxidation. Insulin resistance in skeletal muscle and impaired insulin-induced glucose uptake by skeletal muscle are the key features in T2DM pathogenesis [40]. The GLUT4 translocation is a glucose uptake rate-limiting step and is related to insulin resistance in skeletal muscle [41]. The larger the GLUT4 protein at the cell surface, the greater insulin sensitivity and glucose clearance activity is detected. GLUT4 translocation malfunction in

skeletal muscle in reaction to insulin stimuli is an initial phase to progress insulin resistance and T2DM [42]. BA extracts, specifically BCE, significantly increased skeletal muscle GLUT4 compared with the diabetic control. These results showed that BCE boosted GLUT4 translocation and glucose uptake.

Conclusion

The results of this study suggested that BA extracts showed hypoglycemic effect, hypolipidemic effect, and insulinotropic effect. BA extracts inhibited oxidative stress which led to inhibition of the SAPK-JNK pathway and possibly led to decrease apoptosis in β-cells. Further research is required to test the use of the pure balanitin-2 as a therapeutic intervention against diabetes.

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Conflict of interest

No conflict of interest to be declared.

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Figure Legend

Figure (1): LC-HRMS profiles of (A) crude extract, (B) butanol and (C) dichloromethane fractions of BA.

Figure (2): Histopathological features of pancreas and liver of all studied rat groups.
A-F: pancreatic cells of rats. Haemotoxylin and Eosin (H&E) general stain. Magnification × 1000. (A) Normal control group with normal pancreatic islets containing normal cells. (B) STZ administered group showed a noticeable reduction in both sizes of the islets and number of cells. Note severe degenerative changes in the islets' cells. (C) BCE group showed pancreatic islets look like normal with few degenerative changes in cells. (D) BBF group showed pancreatic islets normal in size and number of cells with few degenerative changes. (E) BDF group showed pancreatic islets smaller in size and fewer cells than normal with more degenerative changes. (F) Gliclazide group showed pancreatic islets smaller in size and fewer cells than normal with more degenerative changes. G-L: Pancreatic cells of rats. Aldehyde Fuchsine stain. Magnification × 1000. (G) Control group showed normal β-cells of islets containing strongly stained purplish granules of insulin. (H) STZ group the islets appear with no β-cells or any purple granules. (I) BCE group showed pancreatic islets containing few β-cells strongly stained with purplish granules (arrow). (J) BBF group showed strongly stained granules in β-cells look like normal (arrow). (K) BDF group showed faintly stained granules in β-cells. (L) Gliclazide group showed faintly stained granules in β-cells. M-R: Liver cells of rats. Periodic acid-Schiff (PAS) stain. Magnification × 400. (M) Control group showed hepatocytes containing very strong fuchsinophilic granules. (N) STZ group showed a noticeable reduction in fuchsinophilic granules in the hepatocytes. (O) BCE group showed strong fuchsinophilic granules in the hepatocytes. (P) BBF group showed hepatocytes containing from moderate to strong fuchsinophilic granules. (Q) BDF group showed moderate fuchsinophilic granules in the liver tissue. (R) Gliclazide group showed weak fuchsinophilic granules in the hepatocytes.

Table (1): LC-HRMS analysis of different extracts of BA.

R_t min	HRMS	Formula	Tentative identification*	Crude	BuOH	DCM
8.40	1047.5371	$C_{51}H_{82}O_{22}$	spirost-5-en-3β-ol 3-O-α-L-rhamnopyranosyl (1->2)-β-D-glucopyranosyl (1->3)-β-D-glucopyranosyl (1->4)-β-D-glucopyranoside	+	+	+
8.96	1087.5315	$C_{53}H_{82}O_{23}$	camellioside C	+	+	+
9.45	1193.5949	$C_{57}H_{92}O_{26}$	26-O-β-D-glucopyranosyl-(25R)-furost-5,20-diene-3,26-diol-3-O-[α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside	+	+	+
10.00	1047.5367	$C_{51}H_{82}O_{22}$	26-O-β-D-glucopyranosyl-(25R)-furost-5,20-diene-3,26-diol3-O-[β-D-glucopyranosyl-(1→2)]-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside	+	+	+
10.45	505.2063	$C_{26}H_{32}O_{10}$	5-[3"-(β-D-glucopyranosyloxy)propyl]-7-methoxy-2-(3',4' dimethoxyphenyl) benzofuran	–	–	+
10.70	1049.5524	$C_{51}H_{84}O_{22}$	trigonelloside C	+	+	+
11.10	1031.5425	$C_{51}H_{82}O_{21}$	balanitin-1	+	+	+
12.10	555.1280	$C_{31}H_{22}O_{10}$	2,3-dihydrobilobetin	+	–	–
12.50	553.1125	$C_{31}H_{20}O_{10}$	Amentoflavone 4'-Me ether	+	–	–
12.92	541.1123	$C_{30}H_{20}O_{10}$	2",3"-dihydroamentoflavone	+	–	+
13.90	1017.5265	$C_{50}H_{80}O_{21}$	balanitin-2	+	+	–
14.28	885.4837	$C_{45}H_{72}O_{17}$	Deltonin	+	+	+
15.40	255.2320	$C_{16}H_{30}O_2$	hexadecenoic acid	+	+	–
16.40	1063.5685	$C_{52}H_{86}O_{22}$	methyl protodioscin	+	+	–
16.64	353.2688	$C_{21}H_{36}O_4$	Tomentol	–	–	+
16.92	1065.5478	$C_{51}H_{84}O_{23}$	26-(O-β-D-glucopyranosyl)-3-β-[4-O-(β-D-glucopyranosyl)-2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranosyloxy]-22,26-dihydroxyfurost-5-ene	+	+	–
17.27	743.4578	$C_{39}H_{66}O_{13}$	2-hydroxycholest-5-ene-3,16-diyl bis(β-D-glucopyranoside)	–	–	+
17.97	497.3106	$C_{27}H_{44}O_8$	pregn-5-ene-3β,16β,20(R)-triol 3-O-β-D-glucopyranoside	+	–	+
18.49	365.2664	$C_{22}H_{36}O_4$	15-acetoxylabd-8-(17)-en-19-oic acid	–	–	+
21.90	431.3153	$C_{27}H_{42}O_4$	Kryptogenin	–	–	+
22.47	415.3208	$C_{27}H_{42}O_3$	Diosgenin	+	–	+
24.70	429.3365	$C_{28}H_{44}O_3$	6-methyldiosgenin	–	–	+
25.90	415.3937	$C_{29}H_{50}O$	Stigmast-4-en-3-ol	–	–	+

* All compounds formulae were calculated using Xcalibur 3.0 software and the suggested compound were identified according to Dictionary of Natural Products (DNP 23.1, 2015 on DVD) and Reaxys online databases.

Table (2): Biochemical analysis in different studied groups.

Parameter	Normal Control	STZ	STZ + BCE	STZ + BBF	STZ + BDF	STZ + Gliclazide
Plasma glucose (mmol/l)	6 ±1.08	25.83±5.93[a]	11.48±0.44[a,b]	8.47±1.65[b]	18.49±3.75[a,b,c,d]	20.17±5.00[a,b,c,d]
HbA_{1c} mmol/mol	20.22±3.21	68.31±5.64[a]	39.01±6.32[a,b]	31.69±8.21[a,b]	46.67±10.36[a,b,d]	57.60±7.19[a,b,c,d,e]
HbA_{1c} (%)	4.00±0.29	8.4±0.51[a]	5.72±0.57[a,b]	5.05±0.75[a,b]	6.42±0.94[a,b,d]	7.42±0.65[a,b,c,d,e]
Plasma insulin (pmol/l)	1423.87±137.60	189.85±20.56[a]	489.19±20.56[a,b]	666.08±96.70[a,b,c]	312.39±20.56[a,b,c,d]	429.52±88.56[a,b,c,d,e]
Plasma Lactic acid (mmol/l)	1.17±.35	4.04±0.93[a]	1.89±0.20[a,b]	1.03±0.07[b,c]	2.13±0.25[a,b,d]	1.86±0.21[a,b,d]
TC (mmol/l)	1.72±0.05	2.59±0.42[a]	1.62±0.20[b]	1.56±0.22[b]	1.63±0.08[b]	1.87±0.25[b]
TG (mmol/l)	2.29±0.18	5.52±0.61[a]	2.94±0.66[b]	2.82±0.18[b]	3.16±.0.79[a,b]	3.08±0.21[a,b]
HDL-C (mmol/l)	1.10±0.05	0.34±0.05[a]	0.89±0.06[b]	0.93±0.07[b]	0.79±0.06[a,b,c,d]	0.54±0.06[a,b,c,d,e]
LDL-C (mmol/l)	0.28±0.05	0.85±0.05[a]	0.19±0.05[a,b]	0.15±0.07[a,b]	0.26±0.06[b,d]	0.99±0.05[a,b,c,d,e]
Atherogenic index (LDL/HDL)	0.25±0.03	2.47±0.27[a]	0.22±0.05[b]	0.16±0.06[b]	0.33±0.09[b]	1.82±0.17[a,b,c,d,e]
MDA (nmol/g liver tissues)	41.20±1.96	77.12±11.96[a]	45.55±3.33[b]	43.20±2.84[b]	48.11±2.82[b]	57.80±2.84[a,b,c,d.e]
Catalase (U/g liver tissues)	989±2	495.62±16.58[a]	954.31±14.92[a,b]	975±2.58[b]	908.31±20.29[a,b,c,d]	744±24.59[a,b,c,d,e]

SOD (U/g liver tissues)	260±29.11	45±3.95[a]	297.50±2.81[b]	375±55.67[a,b,c]	177.72±29.19[a,b,c,d]	255±22.63[b,c,d,e]
GSH (mg/g liver tissues)	64.81±5.53	11.05±2.03[a]	56.66±4.38[a,b]	58.93±5.77[a,b]	50.39±3.03[a,b,c,d]	24.65±4.17[a,b,c,d.e]
ASK1 (µg/g pancreatic tissues)	12.41±2.32	42.71±2.70[a]	23.61±3.42[a,b]	22.73±2.41[a,b]	21.68±5.64[a,b]	21.93±3.87[a,b]
JNK1 (µg/g pancreatic tissues)	47.20±5.20	161.21±6.61[a]	93.47±16.96[a,b]	86±8.51[a,b]	82.27±21.38[a,b]	83.37±14.60[a,b]
P53 (µg/g pancreatic tissues)	17.3±2.62	55.51±3.12[a]	33.01±4.76[a,b]	28.90±3.50[a,b]	30.30±7.86[a,b]	31.13±5.20[a,b]
IRS1 (µg/g pancreatic tissues)	74.60±3.84	12.59±2.49[a]	36.47±6.51[a,b]	29.98±4.28[a,b]	30.39±7.84[a,b]	25.28±8.49[a,b,c]
GLUT4 (µg/g skeletal muscles tissues)	197.31±8.92	76.21±3.80[a]	138.72±15.99[a,b]	114.88±16.70[a,b,c]	89.17±7.64[a,c,d]	122.46±9.36[a,b,c,e]

Values are means ± SD, with the number of animals = 10 for each group.

Using one-way ANOVA followed by Tukey post-hoc test.

[a]$p < 0.05$ versus normal control.

[b]$p < 0.05$ versus STZ group .

[c]$p < 0.05$ versus STZ + BCE.

[d]$p < 0.05$ versus STZ + BBF.

[e]$p < 0.05$ versus STZ + BDF.

HbA_{1c}, glycated hemoglobin; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MDA, malondialdehyde; SOD, superoxide dismutase; ASK1, apoptosis signal-regulating kinase 1; JNK1, c-Jun N-terminal kinase 1; p53, protein 53; IRS1, insulin receptor substrate 1; GLUT4, glucose transporter 4; BCE, Balanites crude extract; BBF, Balanites butanol fraction; BDF, Balanites dichloromethane fraction.

Table (3): Histopathological changes in islets of Langerhans and β-cells of rats' pancreas stained with H&E based on scoring severity of injury*

Group	Diameter of pancreatic islets (µm) Mean ± SD	No. of β-cells/field Mean ± SD	Degenerated cells/field	Vacuolated cells/field	Necrosis	Congestion
Normal Control	1435.50 ± 134.58	18.76 ± 3.36	0	0	0	0
STZ	488.25 ± 58.03[a]	4.08 ± 0.43[a]	4	3	3	3
STZ + BCE	1035.00 ± 315.81[a,b]	13.16 ± 3.47[a,b]	2	1	2	2
STZ + BBF	1152.00 ± 204.81[a,b]	17.96 ± 2.72[b,c]	1	1	0	0
STZ + BDF	881.50 ± 83.18[a,b,d]	10.40 ± 2.69[a,b,c,d]	2	2	1	2
STZ + Gliclazide	606.75 ± 77.58[a,c,d,e]	5.04 ± 0.46[a,c,d,e]	2	2	1	1

Using one-way ANOVA followed by Tukey post-hoc test.

[a]$p < 0.05$ versus normal control.

[b]$p < 0.05$ versus STZ group .

[c]$p < 0.05$ versus STZ + BCE.

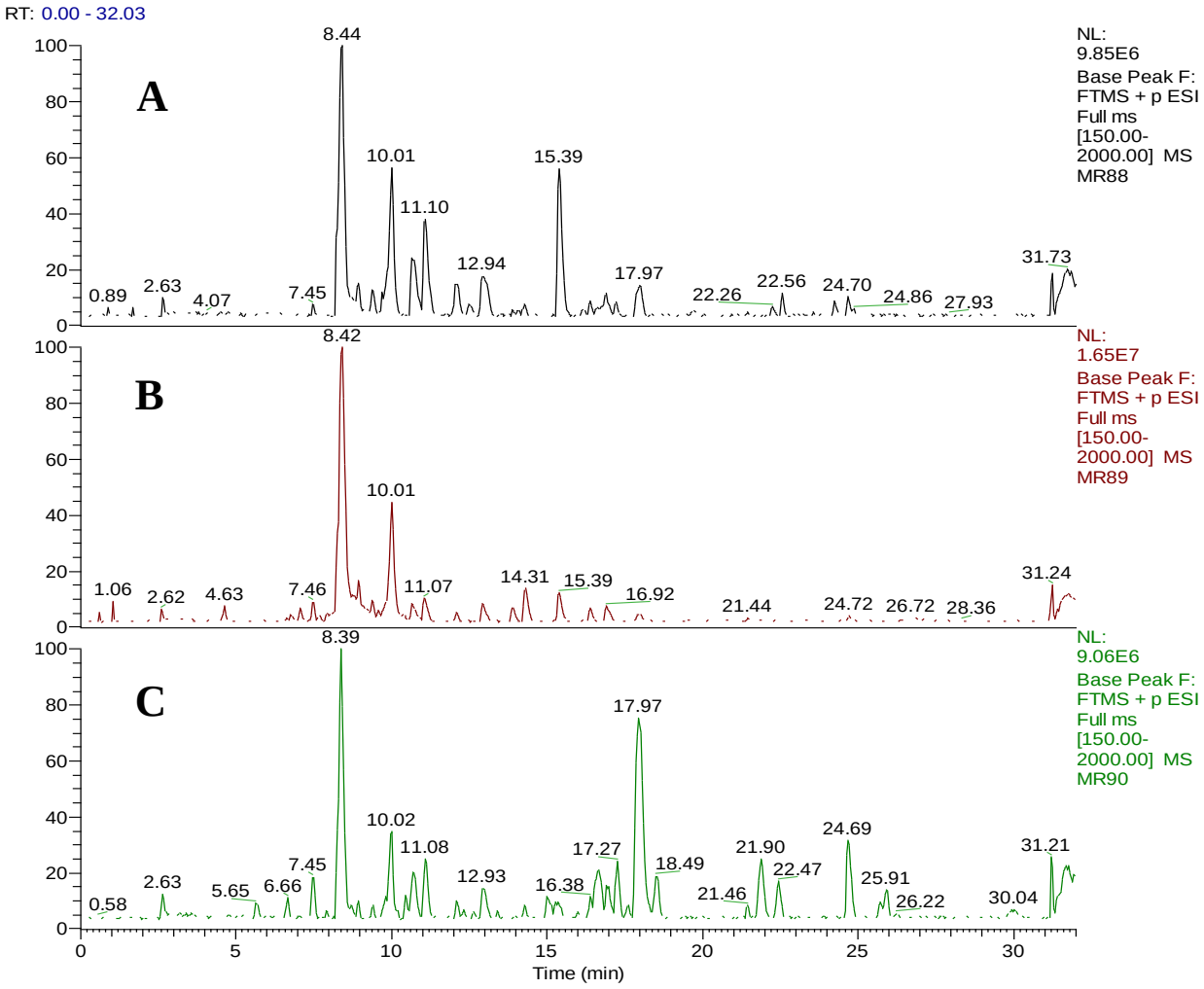
[d]$p < 0.05$ versus STZ + BBF.

[e]$p < 0.05$ versus STZ + BDF.

BCE, Balanites crude extract; BBF, Balanites butanol fraction; BDF, Balanites dichloromethane fraction.

* Tissue injury in β-cells of pancreas was scored in terms of degree of cell damage as follows:

0 = no change; 1 = < 25% cell damage; 2 = 26 – 50% cell damage; 3 = 51 – 75% cell damage; 4 = 76 – 100% cell damage.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9.85E6
Base Peak F:
FTMS + p ESI
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MR88
8.44
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8.42
10.01
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2.62
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Full ms
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8.39
17.97
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11.08
24.69
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7.45
18.49
22.47
2.63
12.93
25.91
5.65
6.66
16.38
21.46
26.22
0.58
30.04
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20
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5
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25
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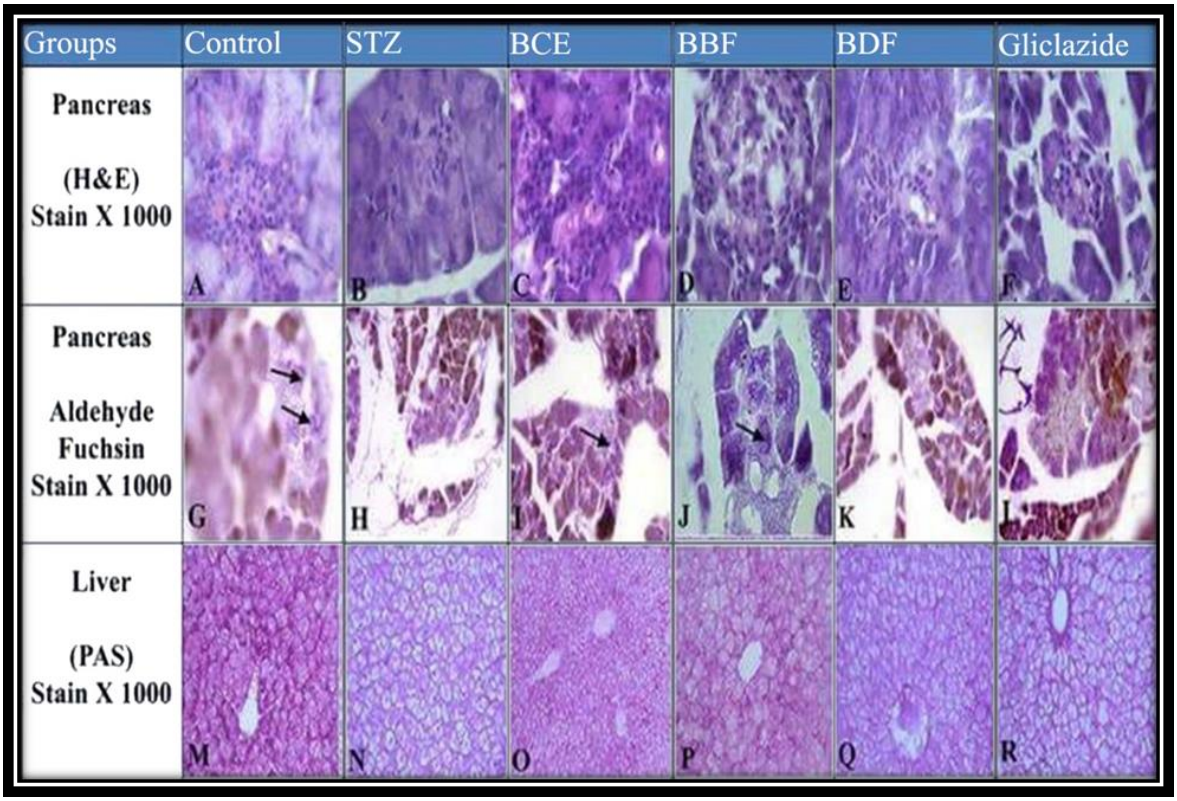
Time (min)

Figure 1


Groups
Control
STZ
BCE
BBF
BDF
Gliclazide
Pancreas
(H&E)
Stain X 1000
A
B
C
D
E
F
Pancreas
Aldehyde
Fuchsin
Stain X 1000
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Liver
(PAS)
Stain X 1000
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Figure 2